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Abstract: **BACKGROUND:** Detection of C4d along peritubular capillaries (PTC) in renal allograft biopsies is an independent prognostic marker of poor long-term graft survival. It is typically associated with circulating donor-specific antibodies. Since only little information is available on the best technique to stain C4d, we compared the two methods most often used for detecting C4d in renal allograft specimens. **METHODS:** We investigated the expression of C4d along PTC in 64 renal allograft biopsies using a monoclonal antibody (Quidel) and immunofluorescence for frozen (F-IF) and a polyclonal antibody (Biomedica) and immunohistochemistry for formalin-fixed and paraffin-embedded (P-IHC) tissue samples. We compared the staining extent (diffuse, focal, minimal, no staining) in frozen and paraffin sections and evaluated the intra- and inter-observer concordance rates using kappa statistics. In addition, we determined the inter-observer concordance in 240 paraffin-embedded biopsies of a multi-centre study. **RESULTS:** The inter- and intra-investigator concordance rate (kappa = 0.9) of analysing the C4d expression by F-IF was excellent. In contrast, the detection of C4d by P-IHC demonstrated a substantially lower prevalence and extent of C4d expression with a lower intra- and inter-observer concordance rate (kappa = 0.3). Only 69% of diffuse and 13% of focal C4d-expressing cases were in line classified by F-IF and P-IHC. On average, the estimated area of C4d-positive PTC in the diffuse group was 36% lower by P-IHC than by F-IF. The inter-observer concordance rate in paraffin of the 64 renal biopsies and the multi-centre study was good, but not perfect (kappa = 0.57 or 0.67). **CONCLUSIONS:** C4d staining determined on frozen tissue samples using F-IF with a monoclonal antibody appears to be better suited for diagnostic as well as research purposes. Future studies should correlate C4d staining patterns with circulating donor-specific antibodies.

DOI: <https://doi.org/10.1093/ndt/gfl594>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-111756>

Journal Article

Published Version

Originally published at:

Seemayer, Christian A; Gaspert, Ariana; Nicleleit, Volker; Mihatsch, Michael J (2007). C4d staining of renal allograft biopsies: a comparative analysis of different staining techniques. *Nephrology, Dialysis, Transplantation*, 22(2):568-576.

DOI: <https://doi.org/10.1093/ndt/gfl594>

Original Article

C4d staining of renal allograft biopsies: a comparative analysis of different staining techniques

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Abstract

Background. Detection of C4d along peritubular capillaries (PTC) in renal allograft biopsies is an independent prognostic marker of poor long-term graft survival. It is typically associated with circulating donor-specific antibodies. Since only little information is available on the best technique to stain C4d, we compared the two methods most often used for detecting C4d in renal allograft specimens.

Methods. We investigated the expression of C4d along PTC in 64 renal allograft biopsies using a monoclonal antibody (Quidel) and immunofluorescence for frozen (F-IF) and a polyclonal antibody (Biomedica) and immunohistochemistry for formalin-fixed and paraffin-embedded (P-IHC) tissue samples. We compared the staining extent (diffuse, focal, minimal, no staining) in frozen and paraffin sections and evaluated the intra- and inter-observer concordance rates using kappa statistics. In addition, we determined the inter-observer concordance in 240 paraffin-embedded biopsies of a multi-centre study.

Results. The inter- and intra-investigator concordance rate ($\kappa = 0.9$) of analysing the C4d expression by F-IF was excellent. In contrast, the detection of C4d by P-IHC demonstrated a substantially lower prevalence and extent of C4d expression with a lower intra- and inter-observer concordance rate ($\kappa = 0.3$). Only 69% of diffuse and 13% of focal C4d-expressing cases were in line classified by F-IF and P-IHC. On average, the estimated area of C4d-positive PTC in the diffuse group was 36% lower by P-IHC than by F-IF. The inter-observer concordance rate in paraffin of the 64 renal biopsies and the multi-centre study was good, but not perfect ($\kappa = 0.57$ or 0.67).

Conclusions. C4d staining determined on frozen tissue samples using F-IF with a monoclonal antibody

appears to be better suited for diagnostic as well as research purposes. Future studies should correlate C4d staining patterns with circulating donor-specific antibodies.

Keywords: comparison of frozen vs paraffin sections; humoral rejection; method of detection for C4d; renal transplantation

Introduction

The diagnosis of humoral rejection of transplanted kidneys is difficult, since typical morphological changes may be minor or lacking altogether. A breakthrough by Feucht *et al.* [1] overcoming these difficulties was the finding of C4d as an immunological marker of the humoral alloresponse.

C4d is a degradation product of the activated complement factor C4 and can be covalently bound to the endothelium (Figure 1). C4d is recognized as an indirect sign or footprint of an antibody response. Deposition of C4d along peritubular capillaries (PTC) has been found to be a sensitive marker for the antibody-dependent humoral rejection of transplanted kidneys. Detection of C4d in PTC is an independent parameter and related with poor graft outcome [2,3]. In earlier studies, the 1-year graft failure rate was 40% in C4d-positive cases compared with only 10% in C4d-negative controls [1].

Our centre in Basel incorporated the analysis of C4d into the diagnostic evaluation of renal allograft biopsies and clinical patient management more than a decade ago. We followed Feucht's original concept and interpreted positive C4d staining results as markers for an antibody-mediated 'severe' rejection episode [4,5].

Insufficient information is available on the best way to uncover C4d deposits in renal biopsies. In early studies [1] monoclonal antibodies in frozen material were used, but later also the detection of C4d in

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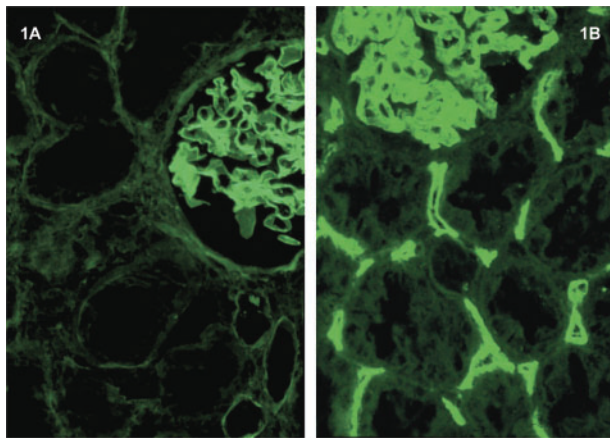


Fig. 1. Expression of C4d of a normal biopsy and a case with humoral rejection as detected by F-IF. (A) Transplanted kidney without rejection: expression of C4d in the glomerulus (mesangium and along basement membrane), but no detection of C4d in PTC. (B) Transplanted kidney with signs of rejection: C4d expression in the PTC of a case with diffuse staining pattern. In addition, the glomerulus is strongly positive for C4d.

paraffin sections by polyclonal anti-C4d antibodies was propagated [6]. Therefore, a purely methodical study was designed to test the two most common anti-C4d antibodies and techniques in use in either frozen or paraffin sections. We would like to emphasize that a correlation of C4d staining patterns with donor-specific antibodies was not in the scope of the study.

Specifically, we have performed a three-level comparison of C4d staining results in PTC in frozen and corresponding formalin-fixed and paraffin-embedded renal allograft biopsies. We used a monoclonal antibody and immunofluorescence microscopy in frozen (F-IF) tissue sections and a polyclonal antibody and immunohistochemistry in formalin-fixed and paraffin-embedded (P-IHC) biopsy material with the following objectives:

- (i) comparison of frozen with frozen biopsies with respect to time-dependent stability and investigator-dependent reproducibility of results (analysed by F-IF)
- (ii) comparison of F-IF and corresponding P-IHC staining patterns
- (iii) investigation of P-IHC staining patterns focusing on the inter- and intra-observer variability in interpreting results.

Materials and methods

Patients and biopsies

Our retrospective analysis included 64 kidney allograft recipients transplanted and clinically managed at the University Hospital of Basel between 1996 and 2004. Allografts were either of cadaveric origin or from living related donors. Biopsies were performed for unexplained

deterioration of graft function. Immunosuppressive therapy of the patients included calcineurin inhibitors, azathioprin, mycophenylate mofetil or steroids.

In addition, renal biopsies from 240 kidney transplant recipients enrolled into a multi-centre study (50 different units, no standardized fixation, no additional clinical information) were also studied. Only formalin-fixed and paraffin-embedded material was available from the latter study group.

Materials

In general, two needle biopsy cores were obtained for morphological work-up. Cores were selected under a dissecting microscope: one for formalin fixation, and the second for quick-freezing in optimal cutting temperature (OCT) embedding medium (Miles Laboratories, Elkhart, IN, USA). The number of glomeruli in the frozen samples was slightly lower than in paraffin-embedded specimens (12.2 ± 5.5 vs 15.8 ± 8.6), but in both groups, sufficient cortex area was available for analysis. The difference is due to our current practice of fixing the larger core for light microscopy, if both cores appear representative under the stereo lens.

Unfixed frozen sections were stained directly (primary diagnostic investigation) for the expression of C4d using immunofluorescence (F-IF1) microscopy and unstained sections were stored airtight at -70°C . The second repeat incubation of the same material for the current investigation was performed after 2.5 years on average (range: 1–8 years). The repeat interpretation of C4d incubations/staining results in frozen tissue samples was performed in a two-step approach: (i) immediate scoring after staining (step 1, F-IF2) and (ii) repeat scoring after extended storage (10–12 months) in a darkroom at 4°C (step 2, F-IF4). The staining results obtained in step 1 were compared with the originally documented diagnostic staining profiles recorded in the patient charts.

Immunofluorescence detection of C4d

Snap frozen tissues were analysed by indirect F-IF technique utilizing a primary affinity-purified monoclonal anti-C4d antibody (mouse anti-human; dilution, 1:50; 30 min incubation at room temperature; Quidel San Diego, CA, USA). The C4d antibody recognizes the antigen of the $\alpha 2$ domain of native C4 and C4d (information provided by the manufacturer). Consecutively, an Alexa-Fluor 488-labelled affinity-purified goat anti-mouse IgG served as a secondary antibody (dilution 1:200; 30 min incubation at room temperature; Molecular Probes Invitrogen, Leiden, The Netherlands). Slides were covered with a specific anti-fading mounting medium according to an adapted protocol from B. Hofer (personal communication). This consists of 2/3 of gelvatol (Air products, Utrecht, The Netherlands; 18.75 g dissolved in 100 ml of 0.5 M Tris buffer at pH 10, plus 1.5 ml of 5% sodium-azide) and 1/3 of a glycerol propyl-gallate mixture (Sigma, St. Louis; 1 g propyl-gallate in 100 ml 50% glycerol). The freshly prepared solution should be mixed for few hours and precipitates are removed by centrifugation (12 000 rpm for 20 min, SS34 rotor). The supernatant can be used directly or stored in the dark at 4°C for about 1 week.

Two pathologists (Investigator 1, C.A.S., Investigator 2, M.J.M.) reviewed independently the biopsies, and the findings were scored according to our standard scoring system (see below).

In addition, frozen biopsies were investigated by a panel of antibodies that recognize various complement factors (C3, C5-9, C4) and immunoglobulins (IgG, IgM, IgA). Specifically, the complement factor C4 was investigated with a polyclonal rabbit anti-C4c antibody (DakoCytomatation) reacting with C4, C4b and C4c, but not with C4d. The expression pattern of C4 obtained with this antibody was compared with the pattern detected by the anti-C4d (Quidel) antibody.

Immunohistochemical detection of C4d in formalin-fixed paraffin-embedded specimens (P-IHC)

C4d can be detected in formalin-fixed and paraffin-embedded tissue sections employing a rabbit polyclonal antibody (C4dpAb, Biomedica, Vienna, Austria) that is specific for human complement split product C4d (aminoacids 1252–1256 of C4) according to the information from the manufacturer. We performed heat antigen retrieval by treatment for 10 min in an autoclave (120°C, 1 bar, in citrate buffer 0.01 M, pH 6.0) for optimal results in our laboratory. In our experience, other pre-treatment protocols, e.g. utilizing a pre-digest with pronase type IV or utilizing a pressure cooker, were less efficient. After antigen retrieval, sections were blocked with anti-avidin and anti-biotin blocking solutions (Zymed Laboratories, distributed by Invitrogen, Switzerland) and with 4% non-fat milk dissolved in phosphate-buffered saline (PBS). The primary antibody was a polyclonal rabbit anti-C4d antibody used at 1:10 and 1:20 dilutions in PBS with overnight incubation at 4°C. Biotinylated goat anti-rabbit antibodies (Vector Laboratories, distributed by Reactolab, Servion, Switzerland) applied for 30 min at room temperature served as secondary antibodies. Subsequently, the signal was amplified by the ABC Elite complex method and with AEC (DAKO, Glostrup, Denmark) serving as substrate for staining. Sections were counter-stained with Mayer's haemalaun for 2 min and finally covered with Pertex mounting medium and cover-slips.

Biopsies obtained in Basel ($n=64$) were interpreted by Investigators 1 and 2. The biopsy specimens of the multi-centre study ($n=240$) were consecutively processed with only one anti-C4d antibody dilution (1:10). This set of biopsies was reviewed by Investigators 2 and 3 (A.G.).

Scoring of the C4d expression in renal allograft biopsies

Biopsies were scored into four categories for the circumferential expression of C4d along PTC in unscarred renal cortex:

- (i) Diffuse expression: >50% of PTC positive.
- (ii) Focal expression: staining of at least 10 PTC and <50%.
- (iii) Focal minimal expression: staining of at least 3 but less than 10 PTC.
- (iv) Negative: staining of less than three PTC or completely negative.

For methodological reasons, a four-step grading system was used to better define the sensitivity of the different detection systems and not for use in daily diagnostic practice.

Renal medulla was not analysed, because medullary tissue had not been obtained in many biopsies. The biopsies of the multi-centre study were scored only to diffuse, focal or no C4d expression in PTC.

In the group of diffusely C4d-expressing cases the percentage area of positive PTC in the unscarred renal cortex was estimated by two investigators (1 and 2) for both frozen and paraffin-embedded parallel sections. In frozen sections, the number of PTC could be analysed by staining additional sections with anti-HLA DR antibodies indicating all PTC. In paraffin sections, counter-staining with haemalaun showed the vessel distribution in unscarred tissue.

All reviewers analysed the slides completely independently from one another. For the analysis of the intra-observer variability (Investigator 2), a time interval of 3 weeks was set.

Statistical analysis

For statistical analysis the software package Jmp (SAS) was used to calculate κ -values (κ -test). κ -values from 0 to 0.2 indicate a poor correlation, from 0.21 to 0.45 a moderate, from 0.46 to 0.75 a good and from 0.76 to 1.0 an almost perfect concordance.

Results

Qualitative comparison of C4d expression in renal allograft biopsies—F-IF vs P-IHC

In normal renal tissues (derived from unaffected areas adjacent to kidney tumours), C4d was expressed in the mesangium of the glomerulus in frozen native specimen but not in paraffin sections [5]. In biopsies from patients with renal transplants (Figure 1) without signs of rejection or glomerular pathology of any kind, the peripheral glomerular basement membrane stained weakly in a linear fashion in some cases by F-IF. In the presence of glomerular damage of any kind (e.g. transplant glomerulitis, glomerulonephritis), a strong C4d staining of the glomerular basement membrane was detected in frozen as well as in paraffin sections. In addition, arteriolar hyalinosis and commonly the thickened tubular basement membrane bound C4d. On occasion, endothelia of the arteries and arterioles were positive for C4d. However, C4d was not found in the tubular epithelial cells or normal tubular basement membranes.

Although only circumferential linear capillary C4d deposits along PTC [1] in the renal cortex were considered as diagnostic staining in our study (Figure 1), a linear staining of PTC in the medulla is as diagnostic as in the cortex. The staining of the PTC in the medulla is often even stronger than in the cortex in both frozen and paraffin-embedded tissues. Especially in cases of extensive cortical necrosis, the staining of PTC in the medulla allows the definite interpretation of the biopsy as positive or negative. However, in the current study we evaluated only

cortical areas, since no medullary tissue was present in many biopsies.

Non-diagnostic or spurious C4d staining was found in foci of arteriolar hyalinosis, thickened tubular basement membranes, protein resorption droplets of the tubules and the tubular brush border or in cellular interstitial infiltrates more frequently in paraffin sections with a polyclonal antibody. Furthermore, C4d-positive plasma in capillaries made the interpretation of PTC staining more difficult in some cases. In paraffin sections granular C4d deposits could overlay the specific linear C4d expression in PTC, thus interfering with the interpretation of the C4d staining pattern. Therefore, different antibody dilutions (1:10 and 1:20) have to be used and compared in the daily diagnostic practice to prevent false interpretation due to artifacts.

In frozen sections, we also compared the expression pattern of anti-C4d (Quidel) with the anti-C4 antibody (DakoCytomation). The latter antibody recognizes the complement products C4, C4b and C4c, but not C4d. Intriguingly, in all our 64 biopsies, the expression of C4, C4b or C4c did not co-localize with the expression of C4d as detected with the Quidel antibody. Specifically, no co-localization was found in the interstitial tubular capillaries of renal allograft biopsies. Accordingly, we assume that the Quidel anti-C4d antibody reacts in our setting only with the C4d split product.

Comparison of C4d expression between frozen and stored frozen biopsies by F-IF

Re-incubation of stored unstained slides. In the group of 64 renal allograft biopsies that were re-incubated after extended airtight storage of unstained slides (on average after 2.5 years) at -70°C for this study, 26 cases revealed a diffuse C4d staining pattern, 23 a focal, six a focal minimal and nine no C4d staining.

The two investigators (1 and 2) agreed completely in their interpretation of the C4d expression in frozen material using a monoclonal antibody (100% concordance). The comparative analysis of the originally recorded diagnostic C4d staining results in frozen biopsy cores with the scoring results of the repeat incubations showed a high concordance rate (Figure 2A, Table 1, $\kappa=0.9$). Only the group with the focal minimal expression of C4d was less frequent in the retrospective setting than in the original diagnostic investigations.

Re-evaluation of stained slides. In addition, we analysed the sections with a diffuse C4d expression pattern stained by F-IF immediately after staining and after storage in a cool darkroom for 10–12 months. Directly after staining, on average $95 \pm 10.6\%$ of the PTC were positive and after storage still $88 \pm 14.6\%$ of the PTC remained positive. Noteworthy, all of the 26 diffuse cases remained 'diffuse' after extended storage of the slides in the dark at 4°C .

Categorical comparison of expression of C4d: F-IF vs P-IHC (Figure 2B and C)

The results of the expression of C4d in PTC detected by F-IF utilizing mouse monoclonal anti-c4d antibodies were set as reference and compared with two dilutions of rabbit polyclonal anti-C4d antibody (1:10, 1:20) in paraffin. For the sake of clarity, only the 1:10 dilution analysed by Investigator 2 is described in detail (for analysis by Investigator 1 and the 1:20 dilution, see Table 1).

Analysis by Investigator 2 in the 1:10 dilution (Table 1). Of 26 cases with a diffuse staining pattern of C4d in PTC as seen by F-IF, only 18 (69%) showed an identical staining pattern by IHC (Figure 2C). The remaining eight cases were scored as focal ($n=6$, 23%) or focal minimal expression of C4d ($n=2$, 8%). Similarly, of 23 renal biopsies with a focal expression of C4d by F-IF, three cases (13%) were concordantly evaluated as focal by P-IHC. From the remaining 20 cases, seven (30%) were evaluated as focal minimal expression and 12 (52%) were considered as negative. Only one case (4%) was upgraded to diffuse C4d expression. Therefore, a shift of the curve of the C4d expression pattern to more negative cases can be seen (Figure 2B).

Accordingly, of the six cases with focal minimal expression as shown by F-IF, only one (17%) was identically scored by IHC. Five cases (67%) differed: four were scored as negative and one (17%) was interpreted as focal C4d positive.

All nine negative cases by F-IF were identically scored indicating an excellent concordance for the truly negative cases. The low agreement between paraffin (1:10 dilution) and frozen sections is also reflected by the statistical analysis. The κ -values of $\kappa=0.34$ (Investigator 1) and $\kappa=0.32$ (Investigator 2) reveal a correlation of only moderate strength. The results were similar for the 1:20 dilutions, but the sensitivity was even lower (Table 1).

Semi-quantitative assessment of C4d expression—F-IF vs P-IHC

In the 26 diffuse C4d cases, we analysed the percentage area of positive PTC and compared frozen and paraffin biopsies (Figure 3). On average, $95 \pm 10.6\%$ of the PTC were positive in the frozen sections. Only six of 26 cases revealed a staining in $<100\%$ (range 60–90%) by F-IF. In contrast, in the corresponding paraffin sections, only $59 \pm 29.4\%$ (1:10 dilution) or $46 \pm 31.6\%$ (1:20 dilution) of all PTC stained positive for C4d. This indicates a loss in sensitivity in recognizing C4d by P-IHC. Noteworthy, of the 20 cases with a 100% diffuse C4d staining pattern in frozen sections, only four (1:10 dilution) or two (1:20 dilution) demonstrated in paraffin also, a 100% C4d positivity in PTC.

If our grading system of the C4d expression in PTC is translated into a numerical score ranging

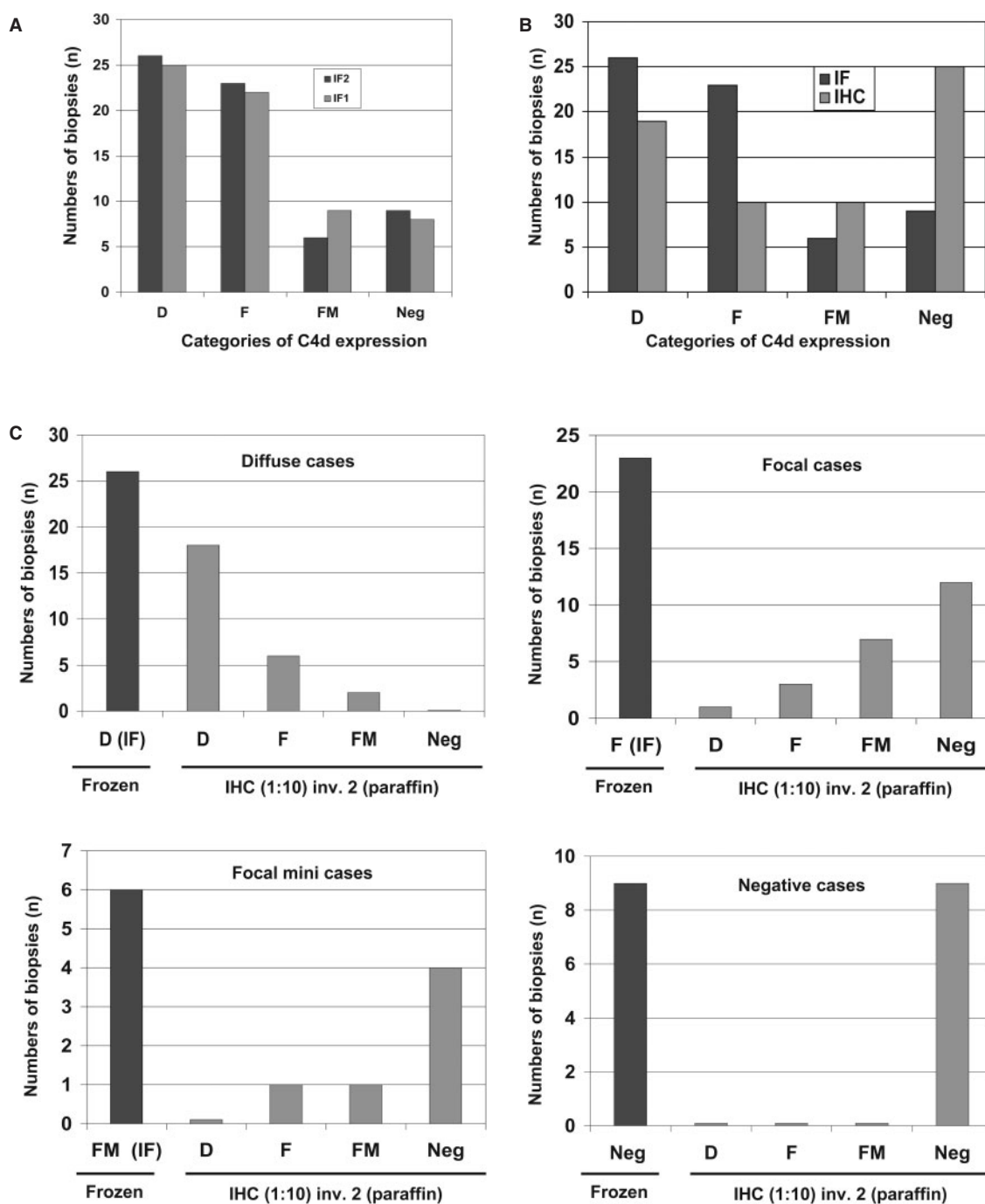


Fig. 2. (A) Comparison of the time-dependent stability of the C4d expression in renal allograft biopsies in F-IF. Unfixed frozen sections were either stained directly (primary investigation, F-IF1) for the expression of C4d or were stored airtight at -70°C . The second investigation (F-IF2) of stored sections was performed in mean after 2.5 years (range 1–8 years). On the x-axis, the categories of the C4d expression in peritubular capillaries are classified as diffuse (D), focal (F), focal minimal (FM) or negative (Neg) staining. (B and C) Categorical comparative analysis of C4d expression between F-IF and P-IHC. (B) The pattern of C4d expression is compared in parallel biopsies between F-IF and P-IHC. Expression of C4d is diagnosed according to the C4d staining pattern in PTC, D, F, FM, Neg). For the sake of clarity, data are only presented for Investigator 2 in the 1:10 dilution. Noteworthy, the prevalence of C4d positivity (diffuse and focal) is higher in the F-IF whereas C4d negative or only focal minimally expressing cases are more often found in the P-IHC group. (C) Detailed analysis of the precise distribution of C4d expression is given for each category. Investigations of F-IF are set as standard (first row) and compared with P-IHC. Results are presented in four plots according to the D, DF, FM and no-C4d expressions. *Note:* Only 69% of the diffuse C4d-expressing cases detected by F-IF were in line with those classified by P-IHC. In contrast, the C4d-negative cases were identically scored for both techniques.

Table 1. Statistical comparisons of the expression of C4d in PTC with respect to different methodological aspects.

Type of comparison	Compared item	κ -value
Frozen with frozen	F-IF1/F-IF2	0.9
	F-IF2 Investigator 1/2	1.0
	F-IF2/F-IF4 (stored)	1.0
Frozen with paraffin	F-IF2/Investigator 1	
	P-IHC ab-dilution	1:10 0.34
		1:20 0.29
	F-IF2/Investigator 2	
	P-IHC ab-dilution	1:10 0.32
		1:20 0.27
Paraffin with paraffin	Investigator 1/2	
	P-IHC ab-dilution	1:10 0.57
		1:20 0.63
	Intra-observer (Investigator 2)	
	P-IHC ab-dilution	1:10 0.68
		1:20 0.83

F-IF, immunofluorescence on frozen sections; P-IHC, immunohistochemistry on paraffin sections; F-IF1, first diagnostic investigation; F-IF2, investigation of sections prepared for this study (mean interval between F-IF1 and F-IF2 = 2.5 years); F-IF2 vs IF4 (stored), evaluation before and after storage of stained slides for 10–12 months in a darkroom.

κ -values from 0 to 0.2 indicate a bad correlation, from 0.21 to 0.45 a moderate, from 0.46 to 0.75 a good and from 0.76 to 1.0 an almost perfect concordance.

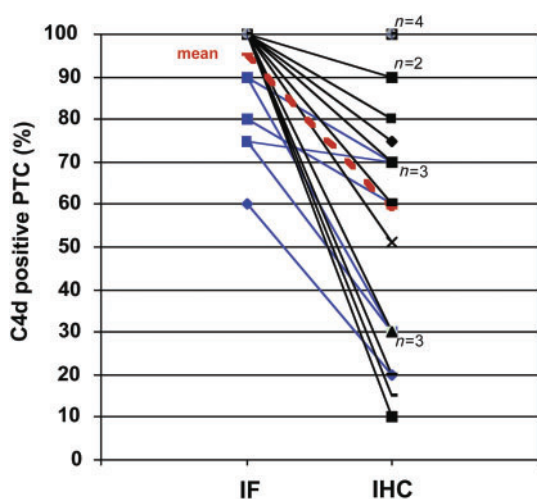


Fig. 3. Numerical comparison of C4d-positive PTC in diffuse C4d staining cases: F-IF vs P-IHC. The number of positive PTC in the group with a diffuse C4d staining pattern ($n=26$) was estimated in percentage by F-IF and compared with the number obtained by P-IHC (dilution 1:10, Investigator 2). Twenty of 26 biopsies revealed by F-IF a 100% diffuse staining pattern (black lines). The remaining six cases (blue lines) demonstrated by F-IF a C4d positivity between 60–90%. Notably, some of the curves are overlapping. For those curves representing more than one case (only black lines), the precise number of entities is given at the right side. The dotted bold line in red indicates the connection between the means of both groups. On average $95\% \pm 10.6$ of the PTC were positive by F-IF, but only $59\% \pm 29.4$ of the PTC by P-IHC, revealing a significant ($P < 0.01$) loss in sensitivity in the second group. Overall, 22 of 26 parallel paraffin biopsies revealed a decrease in the percentage of positive PTC.

from 0 (=negative) to 3 (=diffuse positive) and the mean scores of frozen and paraffin tissues are compared (Δ -values), Investigator 1 would come up to a 0.6 lower and Investigator 2 with a 0.7 lower C4d expression score in paraffin. After exclusion of the negative cases, the discrepancy is even more pronounced with a Δ -value of 0.7 (Investigator 1) and 0.8 (Investigator 2), respectively. This demonstrates that on average, one grade of sensitivity is lost for the IHC detection of C4d in paraffin sections. This corresponds to a shift from diffuse to focal or focal to focal minimal C4d expression.

Comparison of analyses by Investigators 1 and 2 for formalin-fixed paraffin biopsies (P-IHC)

The interpretation of the P-IHC data was more difficult and Investigators 1 and 2 demonstrated a certain degree of discordance (Table 1). For the diffuse C4d cases in paraffin-embedded tissues (interpreter 2 set as reference, dilution 1:10), 16 of 19 cases (84%) were concordant. Similarly, eight of 10 (80%) with focal C4d expression demonstrated the same result. Lower was the rate of concordance in the group of focal minimal C4d expression with two of 10 (20%). The cases with no C4d expression correlated better with an agreement of 18 of 25 between the two observers (72%, dilution 1:10). Slightly higher was the correlation for the 1:20 dilution as reflected by the κ -value.

In general, the inter-observer concordance rate was higher for paraffin sections than the intra-observer comparison of frozen with paraffin sections, but still strikingly lower than the inter-observer concordance in frozen material studied by F-IF. This can also be seen in the higher κ -values for the paraffin with paraffin comparison ($\kappa = 0.57$, dilution 1:10), indicating a good, but not perfect, inter-observer correlation. The most problematic cases were those with focal minimal C4d expression of C4d. If the cases with focal minimal C4d expression were excluded from the analysis, the inter-observer concordance rate increased ($\kappa = 0.67$, 1:10 dilution). Intriguingly, the intra-observer variability (performed only for Investigator 2) for the 1:10 dilution with a κ -value of 0.68 was in a similar range as the inter-observer variability. Also, the intra-observer correlation rose when the focal minimal C4d-expressing cases were not considered ($\kappa = 0.87$, 1:10 dilution). A good correlation for both reviewers was seen for the comparison of the dilution 1:10 and 1:20 by P-IHC with a κ -value of 0.69 or 0.68, respectively. However, the 1:20 dilution in paraffin revealed a higher inter- and intra-observer correlation due to less background/non-specific staining, but was less sensitive than the 1:10 dilution.

Inter-observer variability in interpreting P-IHC for the expression of C4d: a multi-centre study

Two hundred and forty formalin-fixed and paraffin-embedded unselected renal allograft biopsies

of a multi-centre study were analysed for the accumulation of C4d along PTC. These biopsies were stained for the expression of C4d only in the 1:10 dilution and were reviewed by Investigators 2 and 3. The scoring system consisted of a three-step grading, including diffuse, focal or no C4d expression in PTC. Investigator 2 was set as reference.

Thirty-three of the 240 specimens were considered as diffuse C4d expression by Investigator 2, and 30 of them were identically evaluated by Investigator 3 (91%). Lower was the rate of agreement for the focal C4d-expressing entities, resulting in 10 of 18 cases (56%) being consistently diagnosed. In this group, Investigator 2 downgraded three cases (17%) to no C4d expression and upgraded five cases (28%) to diffuse C4d expression. One hundred and sixty-eight of 189 (89%) negative cases were identically scored by both reviewers. As shown by the κ -value of 0.67, overall there was a good correlation, but not perfect inter-observer concordance for the analysis of the cases of the multi-centre study.

This demonstrates that a multi-centre study in C4d staining was feasible and gave similar results as in single-centre studies despite a large variability in the preparation of paraffin sections (e.g. duration of fixation, type of paraffin, temperature during the preparation process).

Discussion

We have performed a three-level approach to analyse the technical- and investigator-dependent variability in detecting C4d expression in PTC of renal allograft specimens: comparisons of (i) frozen with frozen, (ii) frozen with paraffin and (iii) paraffin with paraffin sections. To the best of our knowledge, this is the largest and most comprehensive comparative analysis of the two most often applied staining techniques to detect C4d in PTC reported so far. No attempt was made to correlate C4d staining patterns with donor-specific antibodies.

(i) Our study showed excellent results with respect to the following:

- The rate of concordance of two reviewers in interpreting C4d expression of frozen material by F-IF was perfect (100%).
- After storing untreated frozen sections airtight at -70°C for a mean interval of 2.5 years, the concordance rate remained nearly perfect ($\kappa = 0.9$).
- Using our anti-fading mounting medium, F-IF stained sections could be stored for several months in a cool darkroom without obvious changes in the scoring results.

(ii) Our study showed disappointing results with respect to the following:

- The rate of concordance between parallel sections of F-IF and P-IHC ($\kappa = 0.34$ for reviewer 1, $\kappa = 0.32$ for reviewer 2) was only of moderate strength.
- The inter-observer ($\kappa = 0.57$) and intra-observer ($\kappa = 0.68$) variability for paraffin sections (P-IHC) revealed only a good, but not a perfect correlation.

The expression of C4d in formalin-fixed paraffin-embedded tissues by P-IHC was significantly reduced in comparison with frozen sections analysed by F-IF, resulting in a low concordance rate ($\kappa = 0.3$). For instance, of the diffuse C4d expressing cases by F-IF (dilution 1:10), 30% were scored as focal or focal minimal expression by P-IHC. The estimated percentage area of C4d-positive PTC was 36% lower in paraffin-embedded biopsies. This result is of great importance for the adequate interpretation and comparison of staining results from different studies. On average, the degree of staining in paraffin was lower by about one degree. As a rule of a thumb, diffusely staining cases in frozen sections (F-IF) turned to focally positive in paraffin (P-IHC), and even more pronounced were the differences between F-IF and P-IHC for the focally positive cases. This finding was in line with previous studies (comparing frozen and paraffin-embedded materials) revealing a reduced sensitivity to stain immunoglobulins and complement factors after paraffin embedding [7].

The inter-observer variability of interpreting C4d staining results was significantly higher in formalin-fixed paraffin-embedded sections using a polyclonal antibody than in frozen sections. Notably, the inter-observer variability in paraffin was in the same range as the intra-observer variability, indicating general difficulties in interpreting the result of C4d expression rather than being a result of an individual's difficulty in analysing the result.

The P-IHC investigation of C4d revealed two major problems: (i) reduced sensitivity and (ii) difficulties in interpretation. Therefore, we propose to utilize frozen unfixed material for the detection of the C4d antigen in renal allograft specimens. If paraffin-embedded sections only are available, the interpretation of the result should be performed with caution and the knowledge of a decreased sensitivity of this method. Equivocal diffuse expression of C4d by P-IHC in paraffin may occur. Negative findings for C4d in paraffin do not exclude positive findings in frozen material.

In our study, the higher frequency of C4d detection by the Quidel antibody is not explained by an additional unspecific staining of C4, C4b or C4c by this antibody, because, in parallel sections stained with

a specific anti-C4 antibody (DakoCytomation), no co-localization of the F-IF signals of both antibodies was seen.

Previous studies comparing frozen vs paraffin sections were based on a smaller number of cases. Regele and co-workers [6] used 25 normal native kidneys without any staining in the PTC and only 12 kidney allograft biopsies, of which five stained positive in the PTC both in paraffin and frozen sections. In endomyocardial biopsies, Chantranuwat *et al.* [8] compared F-IF and P-IHC detection of C4d implying a slightly reduced sensitivity for the P-IHC group ($n = 35$ C4d-positive cases).

Most recently, Nadasdy *et al.* [9] published a comparative study for the detection of C4d in PTC of 20 renal allograft biopsies. Similar to our study, the authors compared F-IF using the Quidel antibody with P-IHC utilizing the polyclonal BI-RC4D anti-C4d antibody and an immunoperoxidase technique.

Intriguingly and seemingly in contrast to our results, Nadasdy and co-workers concluded from their data that none of the applied methods appeared to be clearly superior to the others. However, analysing their presented raw data, we find striking differences between F-IF (Quidel) and P-IHC (BI-RC4D). Out of 15 cases with a diffuse C4d expression in PTC analysed by F-IF (Quidel), only 12 expressed C4d by P-IHC (-20% , $\kappa = 0.5$). This is fully in line with the results of the current study indicating a loss of sensitivity for IHC in paraffin of 31% ($n = 26$) in the diffuse C4d-expressing group.

In a series of studies, the C4d status in PTC was correlated with morphological lesions and clinical course. Groups from Basel [4, 5], Boston [10, 11], Oxford [12], Leuven [13], Baltimore [14] and Vancouver [2, 15] worked with frozen material and applied monoclonal antibodies, in the majority the Quidel antibody. In contrast, the groups from Hanover [16], Chicago [17] and Vienna [6] utilized the same polyclonal rabbit antibody (BI-C4D), distributed by different companies, and paraffin-embedded material. Even if the results of most studies (independent of the material and antibodies used) point in the same direction, i.e. higher prevalence of transplant glomerulitis/glomerulopathy, transplant endarteritis and higher risk of graft dysfunction in C4d-positive cases, the results are strictly comparable only for the diffuse C4d-positive cases in both frozen and paraffin sections.

The results from our study clearly demonstrate that all the investigations in paraffin harbour the risk of being less sensitive than studies being performed in frozen material, and must be interpreted with great caution, since they might not have unravelled all potential associations that could be recognized in frozen material.

This could well explain the striking differences in the prevalence of C4d positivity in different studies.

The results of our study warrant also some comments to the grading of C4d in PTC in frozen and paraffin sections. In different studies, the grading of C4d expression varied substantially.

Conclusion

Detection of C4d by F-IF in frozen renal allograft biopsies with a monoclonal antibody is a highly sensitive and, in terms of the stability of the signal after storage, robust and with respect to the inter- and intra-observer variability, a highly reproducible method. In contrast, the P-IHC investigation of C4d with a polyclonal antibody is less sensitive and difficult to interpret. Therefore, we propose frozen unfixed material for the detection of the C4d antigen in renal allograft specimens, whenever possible. Future studies should aim to standardize criteria of C4d positivity and to correlate data on circulating donor-specific antibodies with C4d staining patterns in frozen and paraffin sections.

Conflict of interest statement. None declared.

Acknowledgements. We are very thankful to Mrs Ursula Dürmüller, Rita Epper and Susanne Grieshaber for their excellent technical assistance. We thank Prof. Dres. F. P. Brunner and F. Gudat for critically reviewing of the manuscript.

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Received for publication: 31.5.06

Accepted in revised form: 8.9.06